Developmental Biology 409 (2016) 543–554

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Genomes and Developmental Control

Posterior–anterior gradient of zebrafish *hes6* expression in the presomitic mesoderm is established by the combinatorial functions of the downstream enhancer and 3′UTR

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form
13 November 2015
Accepted 15 November 2015
Available online 17 November 2015

Keywords:
Somite
Hairy-related gene
Expression gradient
Transcriptional regulation
3′UTR
Zebrafish

ABSTRACT

In vertebrates, the periodic formation of somites from the presomitic mesoderm (PSM) is driven by the molecular oscillator known as the segmentation clock. The *hairy*-related gene, *hes6/her13.2*, functions as a hub by dimerizing with other oscillators of the segmentation clock in zebrafish embryos. Although *hes6* exhibits a posterior–anterior expression gradient in the posterior PSM with a peak at the tailbud, the detailed mechanisms underlying this unique expression pattern have not yet been clarified. By establishing several transgenic lines, we found that the transcriptional regulatory region downstream of *hes6* in combination with the *hes6* 3′UTR recapitulates the endogenous gradient of *hes6* mRNA expression. This downstream region, which we termed the PT enhancer, possessed several putative binding sites for the T-box and Ets transcription factors that were required for the regulatory activity. Indeed, the T-box transcription factor (Tbx16) and Ets transcription factor (Pea3) bound specifically to the putative binding sites and regulated the enhancer activity in zebrafish embryos. In addition, the 3′UTR of *hes6* is required for recapitulation of the endogenous mRNA expression. Furthermore, the PT enhancer with the 3′UTR of *hes6* responded to the inhibition of retinoic acid synthesis and fibroblast growth factor signaling in a manner similar to endogenous *hes6*. The results showed that transcriptional regulation by the T-box and Ets transcription factors, combined with the mRNA stability given by the 3′UTR, is responsible for the unique expression gradient of *hes6* mRNA in the posterior PSM of zebrafish embryos.

1. Introduction

Somites, transient epithelial structures of the paraxial mesoderm, are periodically segmented from the anteriormost portion of the unsegmented paraxial mesoderm known as the presomitic mesoderm (PSM). The periodicity of somite formation is governed by an internal molecular oscillator, the segmentation clock, which exhibits cyclic gene expression in the PSM (Hubaud and Pourquié, 2014; Oates et al., 2012; Saga and Takeda, 2001). This oscillatory expression is characterized by a tightly coordinated periodicity that approximates a wave of expression that travels through the PSM in a posterior–anterior direction during somitogenesis. Most of the genes that exhibit cyclic expression in the PSM are associated with the Notch signaling pathway. For example, the *hairy/Enhancer of split*-related genes that encode bHLH transcription factors, such as *c-hairy1* in chickens (Palmeirim et al., 1997), *Hes7* in mice (Bessho et al., 2001), and *her1* and *her7* (*her1/7*) in zebrafish (Henry et al., 2002; Holley et al., 2000), are all central components of the oscillation machinery and have been shown to exhibit oscillatory gene expression patterns in the PSM. The negative feedback loop involving these genes comprises the core oscillator of the segmentation clock (Bessho et al., 2003; Giudicelli and Lewis, 2004). Loss of function or overexpression of these cyclic genes results in disruption of the somite boundaries in mouse (Bessho et al., 2001) and zebrafish (Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002). In the posterior PSM, the cyclic expression of *hairy*-related genes is synchronized among neighboring cells via cell-to-cell interactions that are mediated by the Notch signaling pathway (Horikawa et al., 2006; Riedel-Kruse et al., 2007). Expression domains of the genes of the segmentation

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http://dx.doi.org/10.1016/j.ydbio.2015.11.010
0012-1606/

clock are tightly coordinated by mutually antagonistic gradients between retinoic acid (RA) and fibroblast growth factor (FGF) signaling (Hubaud and Pourquié, 2014; Oates et al., 2012). Whereas the anterior–posterior gradient of RA, which is synthesized by RALDH2 in the anterior PSM and newly formed somites, activates the genes involved in segmentation, the opposing gradient of FGF signaling maintains the immature state and cyclical gene expression in the posterior PSM.

In zebrafish, another *hairy*-related gene, *hes6* (previously named *her13.2*), acts as a hub for oscillators in the segmentation clock (Schröter et al., 2012). Hes6 proteins form a transcriptional repressor complex with other cyclic Her proteins that participate in the periodic repression of the Notch-regulated genes *her1* and *her7* (Kawamura et al., 2005; Trofka et al., 2012). Distinct from other cyclic *hairy*-related genes, such as *her1/7* expressed in the PSM (Henry et al., 2002; Holley et al., 2000), the expression of *hes6* mRNA is static and forms a posterior–anterior gradient, which is regulated by FGF signaling and not by Notch signaling (Kawamura et al., 2005). Thus, *hes6* shows an atypical expression profile among the segmentation genes and is considered to be required for the proper expression of other oscillatory genes in the posterior PSM (Kawamura et al., 2005). However, the molecular mechanisms underlying the gradient of *hes6* expression in the posterior PSM remain unknown.

In the current study, we have identified the transcriptional regulatory region, PT enhancer, downstream of the *hes6* locus that drives the expression of reporter genes in the PSM and tailbud. By establishing stable transgenic lines, we demonstrated that the PT enhancer cannot elicit the posterior–anterior gradient of *hes6* mRNA expression on its own, and that a combined function of the PT enhancer and the 3′UTR of *hes6* was enough for recapitulating the expression gradient. Our results suggest that both the transcriptional regulation and 3′UTR-dependent mRNA turnover is responsible for the gradient of *hes6* mRNA expression in the posterior PSM and tailbud.

2. Materials and methods

2.1. Fish maintenance

Zebrafish (*Danio rerio*) were maintained at 27 °C with a 14-h light/10-h dark cycle. Embryos were incubated at 28.5 °C until they reached the appropriate stages. The morphological features and hours post-fertilization (hpf) were used to stage embryos (Kimmel et al., 1995). All experiments using live fish complied with the protocols approved by the Committee for Animal Care and Use of Saitama University.

2.2. Construction of plasmids

DNA fragment for the promoter of zebrafish *hsp70l*, the coding region of enhanced green fluorescent protein gene (*EGFP*), and the SV40 late polyadenylation signal (SV40pA) were inserted into the vector pTol2-MCS (Nakayama et al., 2013). The constructed vector pTol2-hspEGFP was used as the backbone vector in this study. For pTol2-PT enhancer-hspEGFP, a 1.7-kb DNA sequence downstream of the *hes6* locus was amplified by PCR from the zebrafish bacterial artificial chromosome (BAC), CHORI73-20O6, and inserted upstream of the *hsp70l* promoter in pTol2-hspEGFP. Base substitutions in the T-box and Ets binding sites of pTol2-PT enhancer-hspEGFP were introduced using inverse PCR and confirmed by DNA sequencing.

To construct pCS2+tbx16 and pCS2+pea3, DNA fragments that corresponded to the entire open reading frames of *tbx16* and *pea3*, respectively, were amplified by PCR and inserted into the multicloning sites of the pCS2+ vector. To construct pCS2+tbx16-EnR and pCS2+pea3-EnR, DNA fragments that encoded the T-domain of Tbx16 protein (amino acids 1–260) and the DNA fragments that encoded the Ets domain of Pea3 protein (amino acids 345–432), respectively, were inserted in-frame upstream of the *Drosophila* Engrailed repressor domain in the pCS2+ENG-RD vector (kindly donated by Dr. Mami Matsuo-Takasaki).

2.3. Microinjection into zebrafish embryos

To synthesize capped mRNA, template plasmids were linearized with appropriate restriction enzymes and transcribed with SP6 RNA polymerase using the MEGAscript SP6 Kit (Ambion) according to the manufacturer's protocol. Next, 1–2 nl of the capped mRNA was injected into 1–4-cell-stage embryos.

For reporter gene analyses, 1–2 nl of the reporter gene plasmids or BAC EGFP reporters was injected into the 1–4-cell-stage embryos at a concentration of 10 ng/μl.

2.4. BAC homologous recombination in bacteria SW102

Four zebrafish BAC clones used in this study were purchased from BACPAC Resources Center (http://bacpac.chori.org/). To generate the BAC EGFP reporter, a DNA fragment that contained *EGFP*, 3′UTR and the polyadenylation signal from *bovine growth hormone*, and a kanamycin resistance gene was amplified by PCR as described previously (Kimura et al., 2006). The sequences of primers used are 5′-GAACCCGCCGACCATCTGCCCCCCATTCAAGCGTCCTGAACTGCAGCAATATGGTGAGCAAGGGCGAGG-3′ and 5′-TTCTGTGGGTGATTAAAAGACGTGTTTGCGTACCTTTCTGTCTTTTATTCCAGTTGGTGATTTTGAACTT-3′. The PCR product was inserted into the translation start codon of the first exon of the *hes6* gene in BAC clones via homologous recombination in SW102 bacteria, as described previously (Lee et al., 2001). For the deletion analysis of the CHOR211-20O6 BAC EGFP reporter, the candidate DNA fragment in BAC was replaced with a *beta-lactamase* gene or streptomycin resistance gene via homologous recombination. The correct replacements of the 5′ or 3′ regions in the desired sites were confirmed by PCR and DNA sequencing.

2.5. Generation of transgenic fish line

To generate transgenic fish lines, we used the *Tol2* transposon system (Kawakami, 2005). In brief, the pTol2-based EGFP reporter plasmid (10 ng/μl) was co-injected into fertilized embryos with *Tol2* transposase mRNA (25 ng/μl) and the injected embryos were raised to adulthood. Transgenic fish lines were isolated on the basis of fluorescence expression in the F1 embryos obtained from the injected fish. After mating matured F1 fish with wild-type fish, single integration of the transgenes in the genome was confirmed by showing that the inheritance followed the Mendelian law.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Inoue et al., 2008). DNA fragments were labeled with digoxigenin as a probe. *In vitro* synthesized proteins were prepared using pCS2+tbx16 or pCS2+pea3 as templates with the TnT Quick Coupled Transcription/Translation System (Promega).

2.7. Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes were synthesized with DIG RNA Labeling Mix (Roche Diagnostic) according to the manufacturer's protocol. Whole-mount *in situ* hybridization was performed as described previously (Nikaido et al., 1997).

2.8. Two-color fluorescence in situ hybridization

Two-color fluorescence *in situ* hybridization was performed essentially as described previously (Brend and Holley, 2009b). Images were taken by a laser-scanning confocal microscopy (Olympus FV1000).

2.9. Chemical treatments on zebrafish embryos

SU5402 (Wako) treatment was carried out as described previously (Kawamura et al., 2005; Sawada et al., 2001). Manually dechorionated embryos at 1–2-somite stage were incubated for 8 min at a concentration of 0.2 mg/ml SU5402. After the four-times wash, the treated embryos were subsequently fixed and subjected to whole-mount *in situ* hybridization.

4-Diethylamino benzaldehyde (DEAB; Sigma-Aldrich) was used to inhibit RA synthesis. The embryos were incubated in the medium containing DEAB at a concentration of 10 μM from the sphere stage and fixed at the 8–10-somite stage.

3. Results

3.1. Identification of the PT enhancer of hes6 in zebrafish

To understand the molecular mechanisms underlying the establishment of a gradient of *hes6* expression in the posterior PSM and tailbud, we attempted to identify the transcriptional regulatory region(s) that drive *hes6* expression. We constructed BAC-based *EGFP* reporters to survey several hundred kilobases of the genomic region that contains *hes6*. As shown in Fig. 1A, four BAC clones that encompassed the *hes6* locus were modified by inserting the *EGFP* reporter gene into the first exon of *hes6* via homologous recombination in bacteria (Lee et al., 2001). Although transient expression of the introduced genes in zebrafish embryos tended to be mosaic, with more or less ectopic patterns, injection of the CHORI73-2006 BAC EGFP reporter yielded specific EGFP signals in the somites, PSM, and tailbud at the 6-somite stage (Fig. 1B), which included the endogenous expression of *hes6* (Kawamura et al., 2005). In addition, three other similarly modified BAC EGFP reporters induced comparable expression. Thus, posteriorly restricted expression of the EGFP reporters observed here suggest that the transcriptional regulatory element(s) required for *hes6* expression in the PSM and tailbud are located within the BAC sequences employed.

To functionally dissect the genomic region flanking *hes6*, different DNA fragments in the CHORI73-2006 BAC EGFP reporter were replaced with the fragment with an ampicillin-resistance *beta-lactamase* gene, or a streptomycin resistance gene, via homologous recombination in bacteria. Subsequent reporter analyses showed that a 5.5-kb DNA fragment in the intergenic region between *hes6* and the circadian clock gene, *per2*, was required for the transcriptional regulatory activities that drive EGFP expression in the PSM and tailbud (Fig. 1C). This 5.5-kb region was divided into several DNA fragments, each of which was then inserted upstream of the promoter for the *hsp70l* gene, which exhibits a basal activity in the absence of heat-shock (Blechinger et al., 2002), and was connected to the *EGFP* reporter gene (Fig. 1D). Injection of each reporter construct demonstrated that a 1.7-kb DNA fragment between +2.8 kb and +4.5 kb from the translation start site of *hes6* exhibited enhancer activity and induced expression in the somites, PSM, and tailbud (Fig. 1E). Since this specific expression of EGFP overlapped with endogenous *hes6* expression in the posterior PSM and tailbud, this 1.7-kb DNA fragment between +2.8 kb and +4.5 kb was designated as the PSM and tailbud (PT) enhancer.

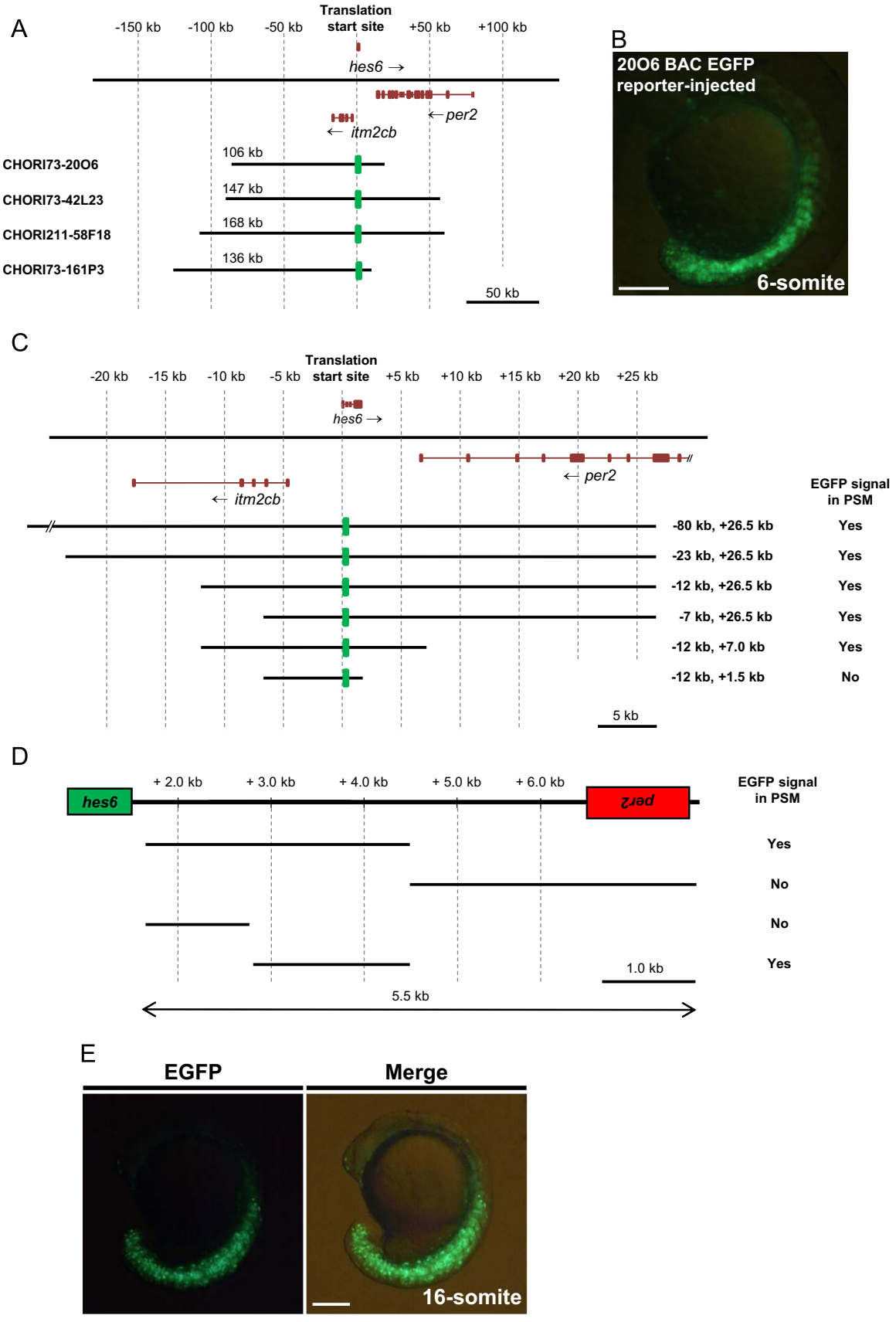
To evaluate the precise regulatory activity of the PT enhancer, we established stable transgenic lines, *Tg(hes6:EGFP-SV40pA)*, which possessed the PT enhancer fused to the *hsp70l*-driven *EGFP* gene and SV40pA, using the *Tol2* transposon system (Fig. 2A). In *Tg(hes6:EGFP-SV40pA)* embryos, the EGFP signal was initially detected in the tailbud around the bud stage (Fig. 2B-E). Relatively strong fluorescence was observed at the posterior end of the notochord in the tailbud (arrowhead in Fig. 2D). Subsequently, at the 18-somite stage, EGFP fluorescence was observed in the somites, PSM, and tailbud (Fig. 2F and G). At 24 hpf, the EGFP signal in the somites appeared to have decreased toward the anterior somites, whereas the fluorescence persisted in the posterior somites (Fig. 2H and I). Next, we compared the expression patterns of *EGFP* mRNA in *Tg(hes6:EGFP-SV40pA)* embryos with the endogenous expression of *hes6* during embryogenesis by whole-mount *in situ* hybridization (Fig. 2J–W). At 50% epiboly, although EGFP fluorescence was not yet detected, a weak *EGFP* mRNA signal consistent with initial endogenous *hes6* expression was observed in the blastoderm margin (Fig. 2J–M) (Kawamura et al., 2005). At the bud stage, the expression patterns of *EGFP* and *hes6* in the PSM and tailbud were comparable (Fig. 2N-Q). At the 12-somite stage, *EGFP* mRNA was again detected in the PSM and tailbud in *Tg(hes6:EGFP-SV40pA)* embryos (Fig. 2R–U). Although *hes6* showed a posterior–anterior expression gradient within the PSM, the expression of *EGFP* did not exhibit any similar gradient; instead, *EGFP* expression was expressed ubiquitously in the PSM and was observed ectopically throughout the somites. At 24 hpf, the *EGFP* signal persisted in the tail region in a manner similar to endogenous *hes6* expression (Fig. 2V–W). A relatively weak signal of *EGFP* mRNA was still observed in the posterior somites, and ectopic expression was also observed in the midbrain and otic placodes. Thus, the expression patterns of *EGFP* in the transgenic fish were generally similar to *hes6* mRNA distribution; however, compared with *hes6* mRNA, *EGFP* expression was more broadly discernible, particularly in somites. Thus, we conclude that the PT enhancer possesses an enhancer activity that is involved in the endogenous expression of *hes6* in the PSM and tailbud, but that the PT enhancer cannot on its own recapitulate the *hes6* expression gradient in the PSM.

3.2. T-box and Ets transcription factor binding sites in the PT enhancer are required for enhancer activity in the PSM and tailbud

To explore the regulatory mechanism of the PT enhancer, putative transcription factor binding sites were identified using the Patch 1.0 program (http://www.gene-regulation.com/pub/programs.html). The putative binding sites for T-box and Ets transcription factors identified with the program were clustered in a 156-bp DNA region at +3.6 kb (Fig. 3A). The two T-box binding sequences in this 156-bp region, located close to each other on opposite DNA strands, were identical to the consensus binding sequence of T-box transcription factors (5′-TCACACCT-3′) and were flanked by three Ets binding sites (Goering et al., 2003; Scott et al., 1994). In zebrafish, several T-box genes such as *ntla*, *tbx6*, and *tbx16* are expressed in the PSM and/or tailbud, and their gene products were shown to bind to this 5′-TCACACCT-3′ *in vitro* or in embryos (Garnett et al., 2009; Morley et al., 2009; Windner et al., 2015). On the other hand, several Ets genes, such as *pea3*, *etv5a*, and *etv5b/erm*, are also expressed in the regions where *hes6* is expressed (Raible and Brand, 2001; Chen et al., 2013). Of note, Ets transcription factors function as downstream mediators of FGF signaling (Raible and Brand, 2001), and the expression of *hes6* is regulated by FGF signaling (Kawamura et al., 2005).

We then examined the effects of substitutions at these binding sites in the PT enhancer (Fig. 3B–L). Injection of the intact PT enhancer-hsp70l-EGFP reporter produced signals in the PSM and tailbud (Fig. 3E and F). By contrast, disruption of the core regions of the two T-box binding sites in the PT enhancer (5′-TCAATCCT-3′,

introduced mutations are underlined) greatly reduced the enhancer activity in the PSM and tailbud (Fig. 3G and H). Mutations in either of the two T-box sites did not reduce the EGFP signal (data not shown), suggesting that each of the T-box sites is sufficient for the enhancer activity. Likewise, base substitutions in the core regions of all three putative Ets binding sites reduced the



enhancer activity in the PSM and tailbud (Fig. 3I and J). Compared to the mutations in the two T-box binding sites, mutations in the Ets binding sites were less effective in decreasing the enhancer activity. Finally, the simultaneous disruption of the two Tbx and three Ets sites greatly diminished the PT enhancer activity (Fig. 3K and L). These results indicate that the cluster of T-box and Ets binding sites in the PT enhancer acts as a core regulatory element and is responsible for the regulatory activity in the PSM and tailbud.

3.3. T-box transcription factor,Tbx16, and Ets transcription factor, Pea3, bind to the PT enhancer in vitro

We showed that the T-box and Ets binding sites are required for the PT enhancer activity in the PSM and tailbud. We therefore performed the EMSA to examine whether the T-box and Ets transcription factors can bind to the PT enhancer. The probes used were the 24-bp DNA fragment (T1) containing one of the T-box binding sites and the 21-bp DNA fragment (E1) containing one of the Ets sites (Fig. 4A). Base substitutions were introduced into the core sequences of T1 and E1 (Goering et al., 2003; Scott et al., 1994). As mentioned above, Ntla, Tbx6, and Tbx16 were shown to bind to the 5′-TCACACCT-3′ (Garnett et al., 2009; Morley et al., 2009; Windner et al., 2015). Among these T-box transcription factors, we examined whether Tbx16 is able to bind to the PT enhancer. Our findings showed that the *in vitro*-synthesized Tbx16 protein indeed bound to T1 (Fig. 4B). In addition, the specificity of binding was verified by abrogation of the shifted bands by excess amount of non-labeled T1 oligo but not by the mutT1 competitor. Next, we investigated whether Pea3, among the several *Ets* genes expressed in the PSM and tailbud (Chen et al., 2013; Raible and Brand, 2001), could bind to the PT enhancer. EMSA showed that the *in vitro*-synthesized Pea3 bound directly to the E1 fragment (Fig. 4C). The specificity of this binding was confirmed by excess non-labeled E1 oligo, but not by mutE1 oligo. These results suggest that T-box and Ets transcription factors bind to the PT enhancer in a specific manner.

3.4. PT enhancer is regulated by T-box and Ets transcription factors in zebrafish embryos

We then examined whether the PT enhancer is regulated by T-box and Ets transcription factors in zebrafish embryos. In general, T-box factors function as transcriptional activators (Conlon et al., 1996). We therefore injected *Tg(hes6:EGFP-SV40pA)* embryos with *tbx16* mRNA and examined the *EGFP* expression. The expression of *EGFP* was detected at 60% epiboly in the blastoderm margin of the control embryos, whereas *EGFP* expression was induced in the *tbx16* mRNA-injected embryos (Fig. 5A and B). We then injected *Tg(hes6:EGFP-SV40pA)* embryos with *tbx16-EnR* mRNA that encoded the T-domain of Tbx16 fused to the Engrailed repressor domain. The expression of *EGFP* was detected at 60% epiboly in the blastoderm margin of the control embryos, whereas *EGFP* expression was greatly reduced in the *tbx16-EnR* mRNA-injected embryos (Fig. 5C and D). These results suggest that the PT enhancer is regulated by T-box transcription factors in embryos.

To examine whether the PT enhancer is also regulated by Ets transcription factors in embryos, we injected *pea3* mRNA into *Tg (hes6:EGFP-SV40pA)* embryos and examined *EGFP* expression at 60% epiboly. Compared with control embryos, the expression of *EGFP* was increased in the blastoderm margin in *pea3* mRNA-injected embryos (Fig. 5E, F), suggesting that Pea3 activates the transcription of *EGFP*. Thus, we fused the Ets-domain of Pea3 to the Engrailed repressor domain (i.e., the *pea3-EnR*). Injection of *pea3-EnR* mRNA into *Tg(hes6:EGFP-SV40pA)* embryos extensively downregulated *EGFP* expression at 60% epiboly (Fig. 5G and H). However, injection of antisense morpholino oligos (MO) specific to *tbx16* and *pea3* respectively did not affect the expression of *EGFP* reporter gene (Supplementary Fig. 1), suggesting the redundancy with other T-box and Ets transcription factors. Overall, these results suggest that the PT enhancer in zebrafish embryos is regulated by the T-box and Ets transcription factors.

3.5. Combination of the PT enhancer and hes6 3′UTR is sufficient to recapitulate the posterior–anterior gradient of hes6 mRNA expression within PSM

Although *EGFP* mRNA was expressed in the PSM and tailbud in *Tg(hes6:EGFP-SV40pA)* embryos, the posterior–anterior gradient in the PSM was not recapitulated and ectopic expression of *EGFP* was observed in the somites (Fig. 2R–U). We assumed an additional regulatory mechanism that endowed *hes6* with a gradient expression pattern within the PSM. One such candidate is 3′UTR, as a previous report showed that the 3′UTR of zebrafish *her1* can restrict reporter gene expression in the PSM (Brend and Holley, 2009a). To examine this possibility, we replaced the SV40pA in the PT enhancer-EGFP reporter with the 3′UTR of *hes6* (Fig. 2A) and generated stable transgenic lines, *Tg(hes6:EGFP-hes6 3′UTR)*, using the *Tol2* transposon system (Fig. 6A). In *Tg(hes6:EGFP-hes6 3′UTR)* embryos, EGFP fluorescence was observed in the somites, PSM, and tailbud in a manner similar to *Tg(hes6:EGFP-SV40pA)* embryos at the 18-somite stage (Supplementary Fig. 2). Next, we compared the *EGFP* mRNA expression in *Tg(hes6:EGFP-hes6 3′UTR)* embryos with those in *Tg(hes6:EGFP-SV40pA)* embryos (Fig. 6B–O). In both transgenic lines, the expression of *EGFP* mRNA was observed in the blastoderm margin at 50% epiboly (Fig. 6B–E) and in the tailbud at the bud stage (Fig. 6F–I). During somitogenesis, *EGFP-SV40pA* mRNA was detected in the PSM, tailbud, and somites (Fig. 6J, L and N), whereas the distribution of *EGFP-hes6 3′UTR* mRNA in *Tg(hes6: EGFP-hes6 3′UTR)* embryos was restricted to the PSM and tailbud, and exhibited a posterior–anterior gradient within the PSM (Fig. 6K, M and O). It should be noted that similar expression of *EGFP* mRNA was observed in embryos from two different founders for either of *Tg(hes6:EGFP-SV40pA)* and *Tg(hes6:EGFP-hes6 3′UTR)*. These observations suggest that the differential reporter expression patterns between *Tg(hes6:EGFP-SV40pA)* and *Tg(hes6:EGFP-hes6 3′UTR)* were not caused by the differences of integration sites of reporter genes, but by the differences of the 3′UTR.

Fig. 1. Identification of the transcriptional regulatory region that drives *hes6* expression in the PSM and tailbud of zebrafish embryos. (A) Schematic representation of the genomic region that encompasses *hes6* in zebrafish. Exons in each gene are shown by dark brown boxes and the arrows indicate the direction of transcription. The numbers above the genomic region represents the distance from the translation start site in *hes6*. The genomic regions encompassed by the four BAC clones used in this study are shown below the genomic region. The insert length of each BAC clone is shown above the bar. The green boxes represent the EGFP reporter inserted in the first exon of *hes6* in each BAC clone. At this scale, the *hes6* gene is represented by a single box; however, *hes6* actually comprises four exons. (B) Injection of the CHORI73-2006 BAC EGFP reporter at a concentration of 10 ng/μl induced EGFP signals in the PSM and tailbud regions at the 6-somite stage; lateral view. (C) Dissection of the flanking genomic region of *hes6* from the CHORI73-2006 EGFP BAC reporter via BAC homologous recombination. The genomic regions encompassed by the deleted BAC clones are indicated at the right of each bar. Each modified BAC clone was injected into embryos at a concentration of 10 ng/μl and the presence of the EGFP signal in the PSM and tailbud was examined at the 10–15-somite stage. (D) Deletion analyses of the intergenic region between *hes6* and *per2*. Each genomic DNA was isolated and inserted upstream of the *hsp70l-EGFP* reporter. The constructed plasmids were injected into embryos at a concentration of 10 ng/μl. Enhancer activities in the PSM and tailbud were examined at the 10–15-somite stage. (E) The 1.7-kb DNA fragment, located 2.8-kb downstream of the *hes6* translation initiation site, was inserted upstream of the *hsp70l-EGFP* reporter. Injection of this *EGFP* reporter gene at a concentration of 10 ng/μl produced specific EGFP signals in the PSM and tailbud at the 16-somite stage. Scale bars in B and E are 200 μm.

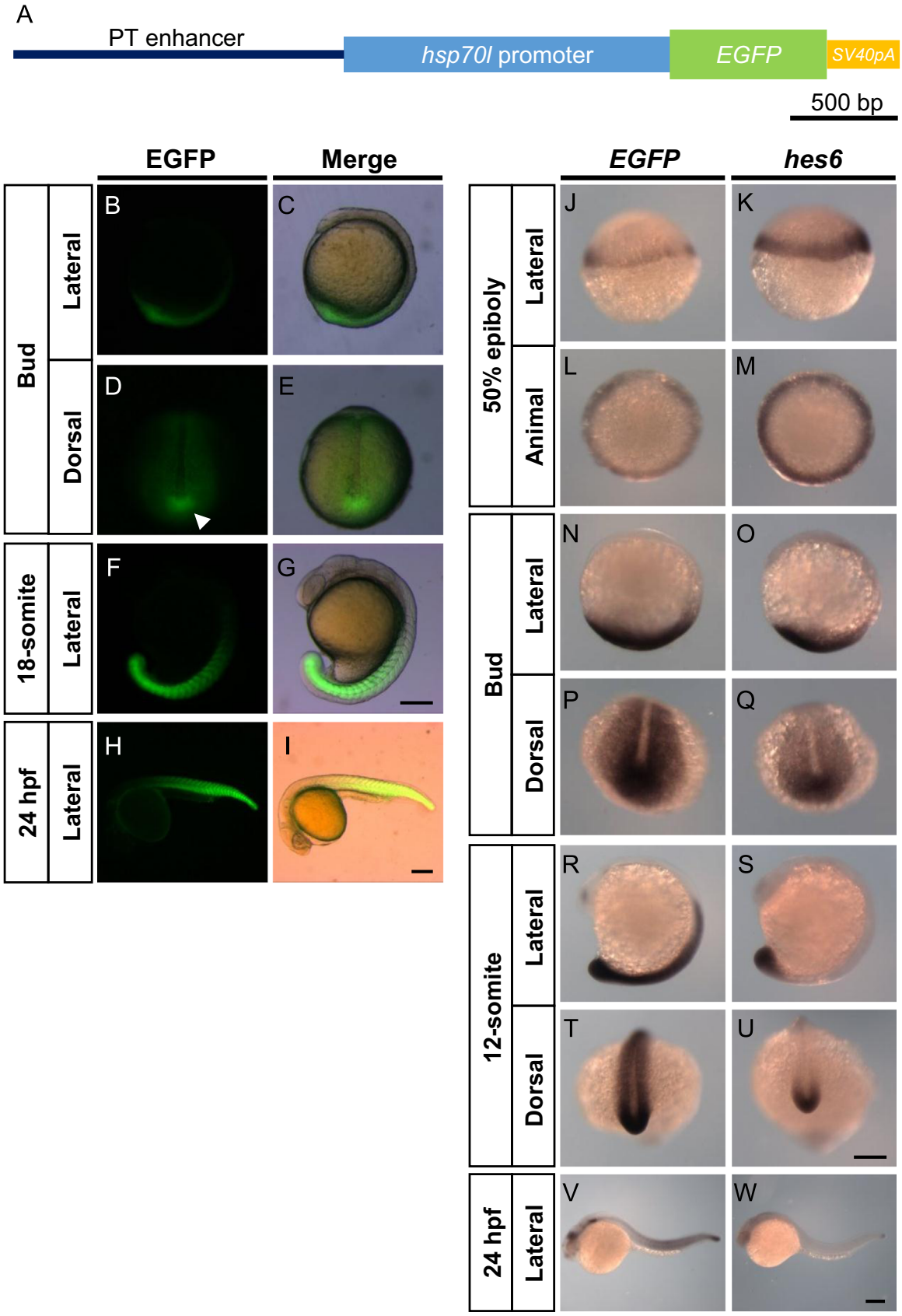


Fig. 2. The PT enhancer-EGFP reporter drives expression in the somites, PSM, and tailbud in transgenic embryos. (A) Reporter gene used to generate the transgenic fish line *Tg(hes6:EGFP-SV40pA)*. The identified 1.7-kb region (PT enhancer) was inserted upstream of the promoter of *hsp70l*, *EGFP* gene, and SV40pA. (B–I) EGFP signals in *Tg(hes6:EGFP-SV40pA)* embryos during development. EGFP expression was initiated in the tailbud at the bud stage. The arrowhead in D indicates the posterior end of the notochord in the tailbud. During somitogenesis, EGFP signal was observed in the somites, PSM, and tailbud. (J–W) Comparisons of *EGFP* reporter expression with endogenous distribution of *hes6* mRNA in *Tg(hes6:EGFP-SV40pA)* embryos. Whole-mount *in situ* hybridization was performed using either *EGFP* or *hes6* antisense probes. Scale bars, 200 μm.

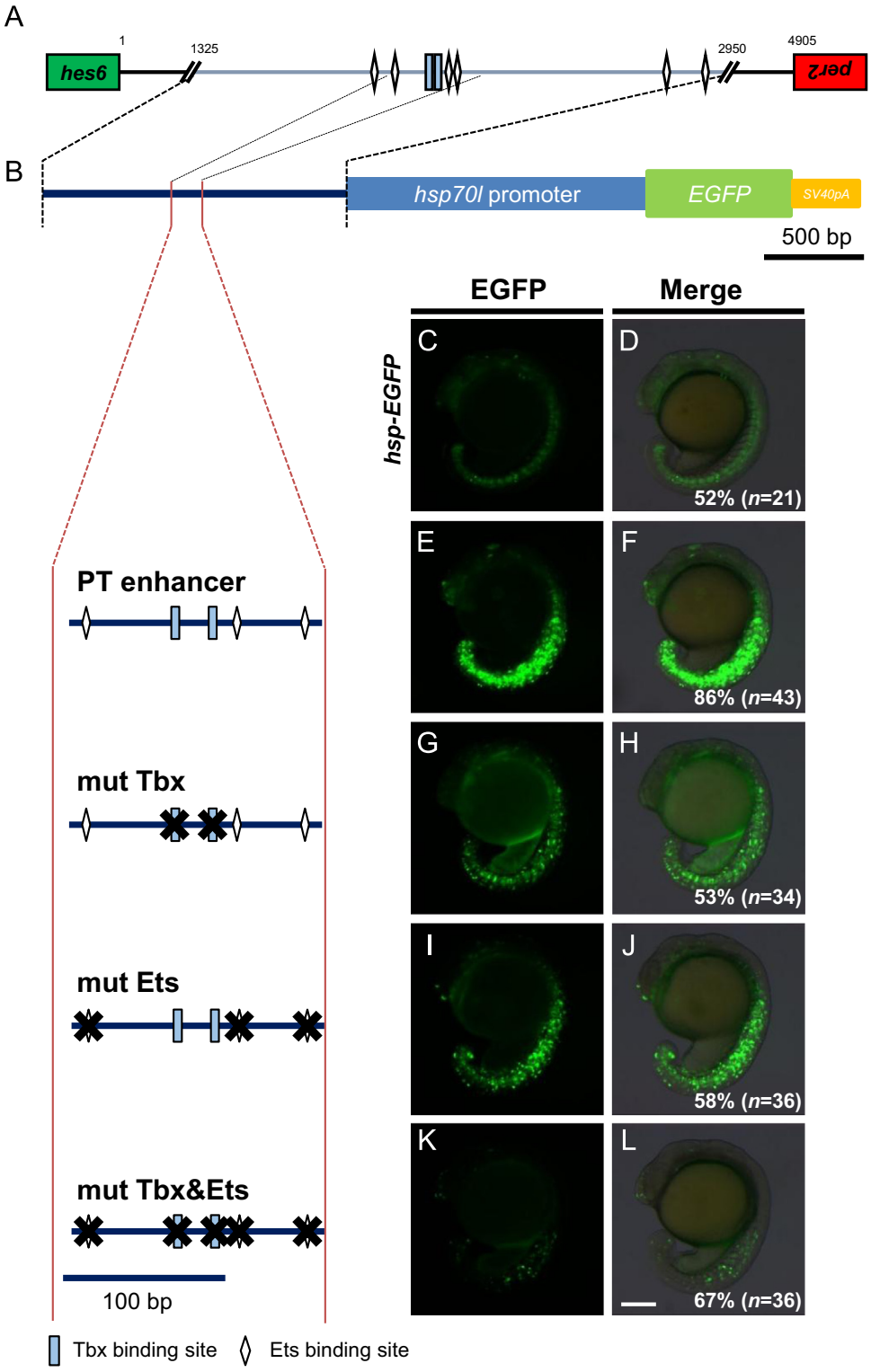


Fig. 3. Binding sites of T-box and Ets transcription factors are required for transcriptional regulatory activities of the PT enhancer. (A) Cluster of putative T-box and Ets transcription factor binding sites in the PT enhancer identified using the Patch 1.0 program. Rectangles represent T-box binding motifs. Diamonds indicate putative Ets binding sites. The green box represents the last exon of *hes6*, whereas the red box is the last exon of *per2*. The orientation of transcription in *hes6* and *per2* is the tail-to-tail opposite in zebrafish. The numbers shown above the bar indicates the base pair distance from the first intergenic sequence between *hes6* and *per2*. (B) The mutations introduced into the T-box and/or Ets binding sites in the PT enhancer ligated to the *hsp70l*-EGFP reporter (shown at top) are shown at left. Several mutated reporter genes were injected into fertilized eggs at a concentration of 10 ng/μl and the EGFP signals in the PSM and tailbud were examined at the 15–18-somite stage, shown at right. The percentage of embryos showing the expression patterns of *EGFP* shown in the panels relative to those of injected normal embryos (*n*) is shown at the bottom-right of each panel. All images were taken at the same magnification. Scale bar, 200 μm.

To precisely compare the expression patterns of *EGFP-hes6 3′ UTR* and endogenous *hes6* mRNAs, we next performed two-color fluorescence *in situ* hybridization at the 10–12-somite stage. Although the expression of *EGFP-SV40pA* mRNA was not restricted to the PSM, but extended to the somites, the distribution of *EGFP-hes6 3′UTR* mRNA clearly exhibited a posterior–anterior gradient

that coincided with that of endogenous *hes6* mRNA expression restricted to the PSM (Fig. 7B–G). Importantly, the anterior limit of the expression domain was comparable between *EGFP-hes6 3′UTR* and *hes6* mRNAs in *Tg(hes6:EGFP-hes6 3′UTR)* embryos. These findings showed that the combination of the PT enhancer and 3′ UTR of *hes6* is capable of recapitulating the endogenous gradient expression of *hes6* in the PSM.

We also generated another transgenic line, *Tg(hes6:EGFP-hes6 3′UTR-SV40pA)*, that possessed the PT enhancer fused to the *hsp70l*-driven *EGFP* gene and both of the 3′UTRs of *hes6* and SV40pA (Fig. 7A) and examined the expression of the reporter gene. Although *EGFP-hes6 3′UTR-SV40pA* mRNA was a little more anteriorly expressed than the mRNA of the endogenous *hes6*, the expression pattern of *EGFP-hes6 3′UTR-SV40pA* mRNA was similar to that of *EGFP-hes6 3′UTR* mRNA, but not to that of *EGFP-SV40pA* mRNA (Fig. 7B-J). In addition, we performed heat-shocks and examined the decay of the induced *EGFP* mRNAs in *Tg(hes6:EGFP-SV40pA)*, *Tg(hes6:EGFP-hes6 3′UTR)*, and *Tg(hes6:EGFP-hes6 3′UTR-SV40pA)* embryos (Supplementary Fig. 3). Whole-mount *in situ* hybridization and quantitative PCR revealed that *EGFP-hes6 3′UTR* and *EGFP-hes6 3′UTR-SV40pA* mRNAs showed a similar rate of mRNA turnover. These results indicate that the *hes6* 3′UTR endows the mRNA with turnover rates required for accurate replication of the endogenous spatial expression profile.

3.6. Expression of EGFP in Tg(hes6:EGFP-hes6 3′UTR) responds to treatments affecting FGF and RA signaling in a manner similar to endogenous hes6 expression.

Along the PSM, antagonistic gradients of FGF signaling and RA control the expression of genes involved in a segmentation clock (Hubaud and Pourquié, 2014; Oates et al., 2012). Indeed, *hes6* expression was shown to be controlled by FGF signaling (Kawamura et al., 2005; Stulberg et al., 2012). To investigate whether *EGFP-hes6 3′UTR* mRNA is also regulated by FGF signaling, we compared the effects of transient treatment with SU5402, a chemical inhibitor of FGF signaling, on *EGFP* expression in *Tg(hes6:EGFP-hes6 3′UTR)* at the 1–2-somite stage. As was observed previously (Kawamura et al., 2005), transient exposure to SU5402 at the 1–2-somite stage reduced the expression of *hes6* mRNA in the PSM of embryos (Fig. 7M and N). Consistent with the endogenous *hes6* expression, the expression of *EGFP-hes6 3′UTR* mRNA was significantly reduced in the PSM by SU5402 treatment compared to the control embryos (Fig. 7K and L).

To examine whether RA also regulates the gradient of *hes6* expression, we treated the embryos using DEAB, a chemical inhibitor of RA synthesis. Treatment with DEAB from the sphere stage resulted in anterior expansion of *hes6* mRNA compared with the control embryos at the 10–12-somite stage (Fig. 7Q and R). These results suggest that the gradient of *hes6* expression in the PSM is regulated by RA. Then, we investigated whether *EGFP-hes6 3′UTR* mRNA is also regulated by RA. Of note, DEAB treatment

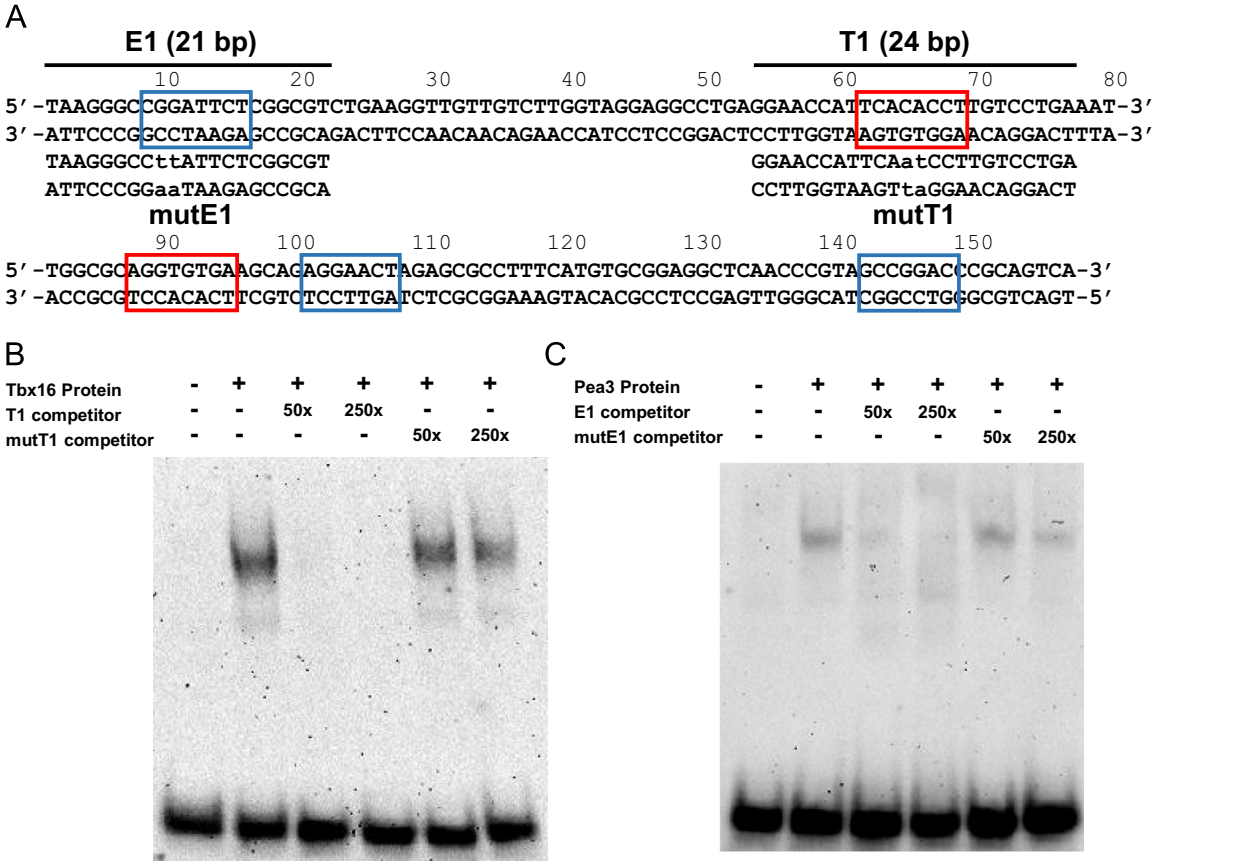


Fig. 4. *In vitro* binding activities of Tbx16 and Pea3 with the core regulatory region of the PT enhancer. (A) The core regulatory sequence in the zebrafish PT enhancer. The T-box binding sites are indicated by red boxes and the Ets binding sites are indicated by blue boxes. The 24-bp DNA fragment that included the T-box binding site was used in EMSA and designated as T1 and the 21-bp DNA fragment that contained the Ets binding site was analyzed in EMSA and designated as E1. Nucleotide substitutions in the mutated forms of T1 (mutT1) and E1 (mutE1) are indicated below the core regulatory sequence in lowercase. (B) EMSA was performed to examine the binding capacities of the *in vitro*-synthesized Tbx16 proteins with the T1 fragment. Binding of Tbx16 with labeled-T1 was inhibited by the presence of 50 fold or 250 fold molar excesses of non-labeled T1, but not by non-labeled mutT1. (C) EMSA analyses showed that the *in vitro* synthesized Pea3 proteins bound to E1 in a specific manner. The binding of Pea3 with E1 was inhibited by the presence of 50 fold or 250 fold molar excesses of non-labeled E1, but not by non-labeled mutE1.

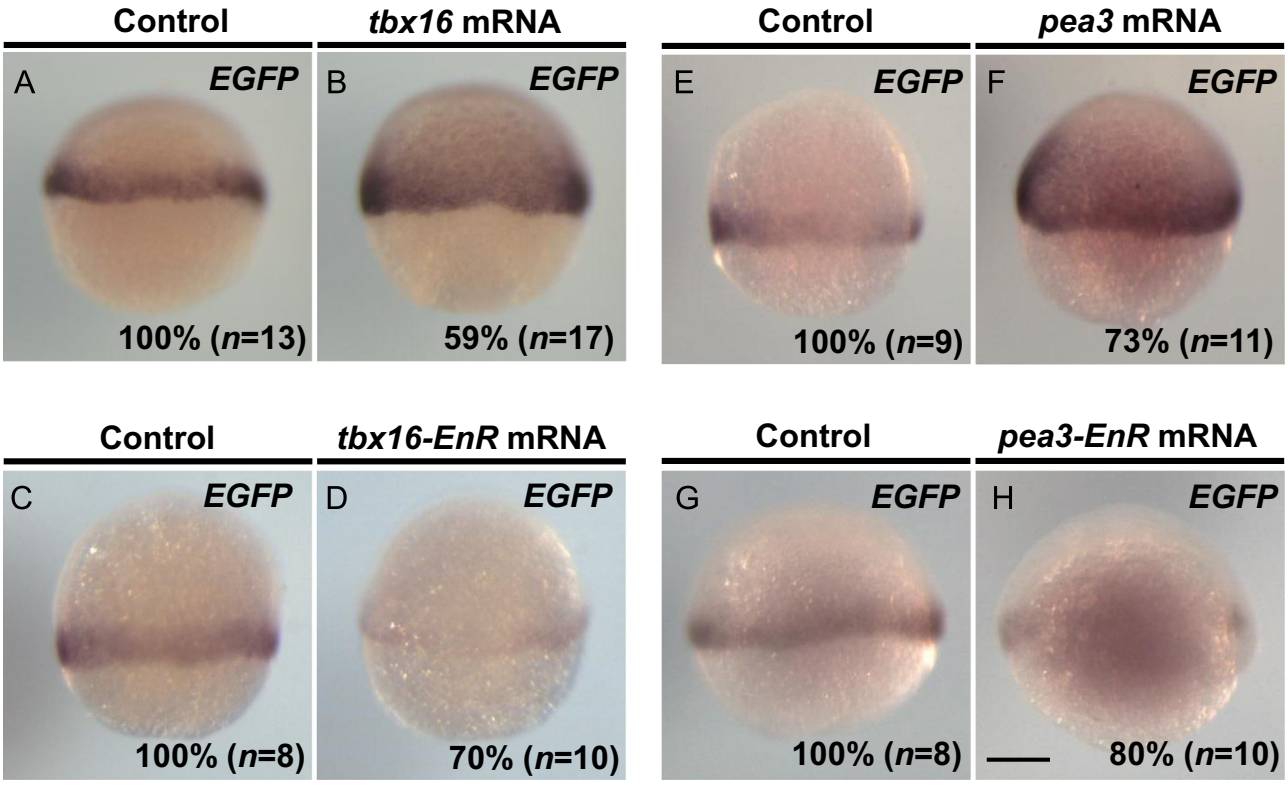


Fig. 5. Enhancer activities of the PT enhancer were regulated by T-box and Ets transcription factors in zebrafish embryos. (A, B) *tbx16* mRNA at a concentration of 200 ng/μl was injected into fertilized embryos produced by crossing *Tg(hes6:EGFP-SV40pA)* and wild type fish. *EGFP* expression was determined by whole-mount *in situ* hybridization at 60% epiboly. (C, D) Injection of *tbx16-EnR* mRNA at a concentration of 100 ng/μl in *Tg(hes6:EGFP-SV40pA)* embryos. *EGFP* expression was determined by whole-mount *in situ* hybridization at 60% epiboly. (E, F) Injection of wild type *pea3* mRNA at a concentration of 100 ng/μl in the *Tg(hes6:EGFP-SV40pA)* embryos. (G, H) Injection of *pea3-EnR* mRNA at a concentration of 100 ng/μl reduced expression of *EGFP* mRNA. *EGFP* expression was examined by whole-mount *in situ* hybridization at 60% epiboly. In all panels, lateral view. The percentage of embryos showing the expression patterns of *EGFP* shown in the panels relative to those of injected EGFP-positive embryos (*n*) is shown at the bottom-right of each panel. All images were taken at the same magnification. Scale bar, 200 μm.

resulted in the anterior expansion of *EGFP-hes6 3′UTR* expression in a manner similar to that of *hes6* (Fig. 7O and P). These results imply that the *EGFP-hes6 3′UTR* recapitulates the altered expression of *hes6* through the inhibition of FGF signaling and RA synthesis. Moreover, our results suggest that the mutually opposed gradients of RA and FGF signaling are translated into the gradient of *hes6* expression.

4. Discussion

In this study, we revealed the transcriptional regulatory sequence downstream of *hes6*, the PT enhancer, which drives gene expression in the PSM and tailbud of zebrafish embryos. We also demonstrated that both T-box and Ets transcription factors bind to the PT enhancer in a specific manner and regulate the enhancer activity. In addition, the posterior–anterior gradient of *hes6* mRNA expression in the PSM is recapitulated by the presence of both the PT enhancer and the *hes6* 3′UTR. Moreover, we also showed that the expression of *EGFP-hes6 3′UTR* mRNA responds to the inhibition of FGF signaling and RA synthesis, respectively, similarly to the endogenous expression of *hes6*. Thus, our results showed that the transcriptional regulation and the mRNA stability given by the 3′UTR are sufficient to replicate the posterior–anterior gradient of *hes6* expression within the zebrafish PSM.

4.1. PT enhancer with hes6 3′UTR recapitulates the endogenous gradient of hes6 mRNA expression in the PSM

The posterior–anterior gradient of *hes6* expression in the PSM is static and is distinct from the expression of other *her* genes, which show cyclical expression. We identified the PT enhancer that drives transcription in the PSM and tailbud. Indeed, in *Tg(hes6:EGFP-SV40pA)* embryos, the expression of *EGFP-SV40pA* was observed in the PSM and tailbud. However, it ectopically extended into the somitic regions, showing that the PT enhancer is not enough for recapitulation of the gradient of *hes6* expression. In contrast, *EGFP* expression in *Tg(hes6:EGFP-hes6 3′UTR)* embryos clearly showed a posterior–anterior gradient in the PSM, as does the endogenous expression of *hes6*. As similar expression patterns of *EGFP* were observed in embryos from different founders for both *Tg(hes6:EGFP-SV40pA)* and *Tg(hes6:EGFP-hes6 3′UTR)* lines, we consider that the ectopic expression of *EGFP* in the somites of *Tg(hes6:EGFP-SV40pA)* is probably attributed to the differences in the stability of *EGFP-SV40pA* and *EGFP-hes6 3′UTR* mRNAs and not to sustained transcription in the somites. In addition, the expression pattern of *EGFP-hes6* 3′UTR mRNA was altered similarly to endogenous *hes6* by the inhibition of FGF signaling and RA synthesis. Although we cannot exclude the possibility that other regulatory element(s) may be involved in the regulation of *hes6* expression in zebrafish PSM, our results indicate that the combinatorial functions of the PT enhancer and *hes6* 3′UTR are sufficient to recapitulate the endogenous spatial expression of *hes6* mRNA in the PSM.

4.2. Identification of PT enhancer suggests transcriptional regulation of hes6 by T-box and Ets transcription factors

We further showed that the putative binding sites for the T-box and Ets transcription factors within the core regulatory sequence of the PT enhancer were required for the enhancer activity. Although FGF signaling was shown to regulate the expression of *hes6*

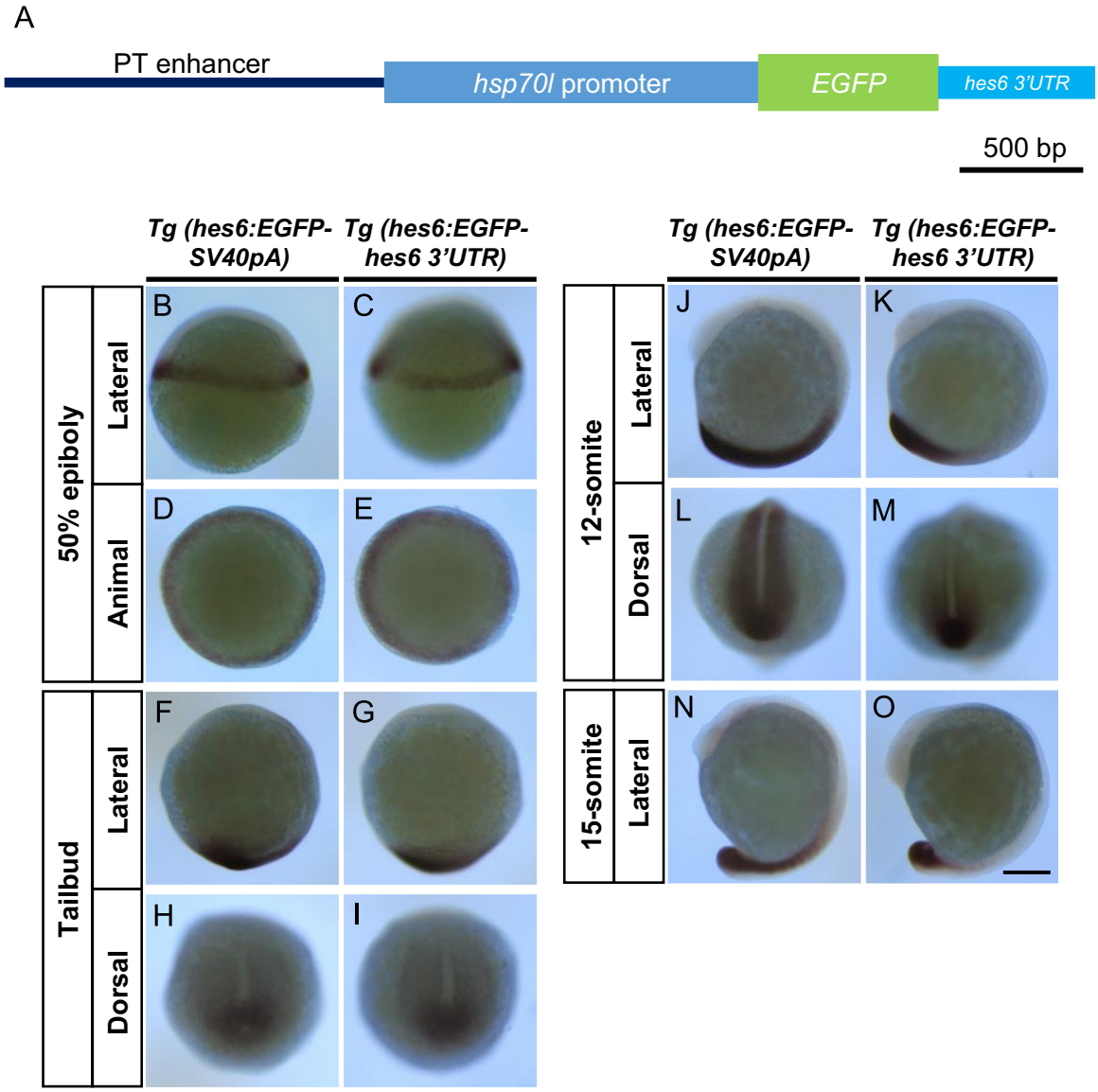


Fig. 6. PT enhancer with *hes6* 3′UTR restricted the *EGFP* mRNA expression to the PSM. (A) *EGFP* reporter genes were used to generate the transgenic fish lines, *Tg(hes6:EGFP-hes6 3′UTR)*. To generate the reporter gene used in *Tg(hes6:EGFP-hes6 3′UTR)*, a DNA fragment corresponding to SV40pA in the reporter (shown in Fig. 2A) was replaced with *hes6* 3′UTR (474 bp). (B–O) Comparisons of *EGFP* mRNA distributions between *Tg(hes6:EGFP-SV40pA)* and *Tg(hes6:EGFP-hes6 3′UTR)* embryos. Scale bar, 200 μm.

in the posterior PSM (Kawamura et al., 2005; Stulberg et al., 2012), the precise molecular mechanism of how FGF signaling controls *hes6* expression has remained unknown. Here, we showed that Pea3, which is a downstream mediator of the FGF/Ras/MAPK pathway (Raible and Brand, 2001), binds to the PT enhancer in a specific manner to regulate the enhancer activity in zebrafish embryos. Zebrafish *pea3* is strongly expressed in the PSM and tailbud, where *hes6* is also expressed. Furthermore, our data are consistent with the previous quantitative study which showed that the nascent RNA of *hes6* is down-regulated by the inhibition of FGF signaling (Stulberg et al., 2012). Therefore, our results suggest that FGF signaling induces the transcription of *hes6* via the induction of Ets transcription factors such as Pea3 in the posterior PSM and tailbud. In addition, we also showed that a T-box transcription factor, Tbx16, can specifically bind to and regulate the PT enhancer. In the PSM of mouse embryos, the expression of the Tbx6 protein, a structural homolog of zebrafish Tbx6 and Tbx16, plays a crucial role in defining the boundaries between future somites (Oginuma et al., 2008). Also in zebrafish, the gene network that is regulated by several T-box genes has been shown to play an important role in somite patterning. For example, Ntla, Tbx6, and Tbx16, regulate the transcription of *her1*, *deltaC*, and *deltaD* which are involved in somite segmentation (Brend and Holley, 2009a; Garnett et al., 2009; Jahangiri et al., 2012). Since knockdown of either *tbx16* or *pea3* did not influence the reporter gene expression, other T-box genes, such as *ntla*, *tbx6*, and *tbx6l*, and Ets genes, such as *etv5a* and *etv5b/erm*, which are also expressed in the PSM and tailbud (Chen et al., 2013; Griffin et al., 1998; Hug et al., 1997; Nikaido et al., 2002; Raible and Brand, 2001), might redundantly regulate the expression of *hes6*.

hes6 expression in the PSM was previously shown to be regulated by FGF signaling (Kawamura et al., 2005), and this study has revealed that expression of *hes6* is also regulated by RA in zebrafish embryos. Cross-regulatory interactions between RA and FGF signaling in the PSM define the future somite boundaries (Hubaud and Pourquié, 2014; Oates et al., 2012). For example, RA signaling activates the expression of *MKP3*, an inhibitor of the MAPK/ERK of the FGF signaling pathway in *Xenopus* (Moreno and Kintner, 2004). Conversely, FGF signaling inhibits RA by activating *CYP26* expression as well as by inhibiting the expression of *RALDH2* in the chick

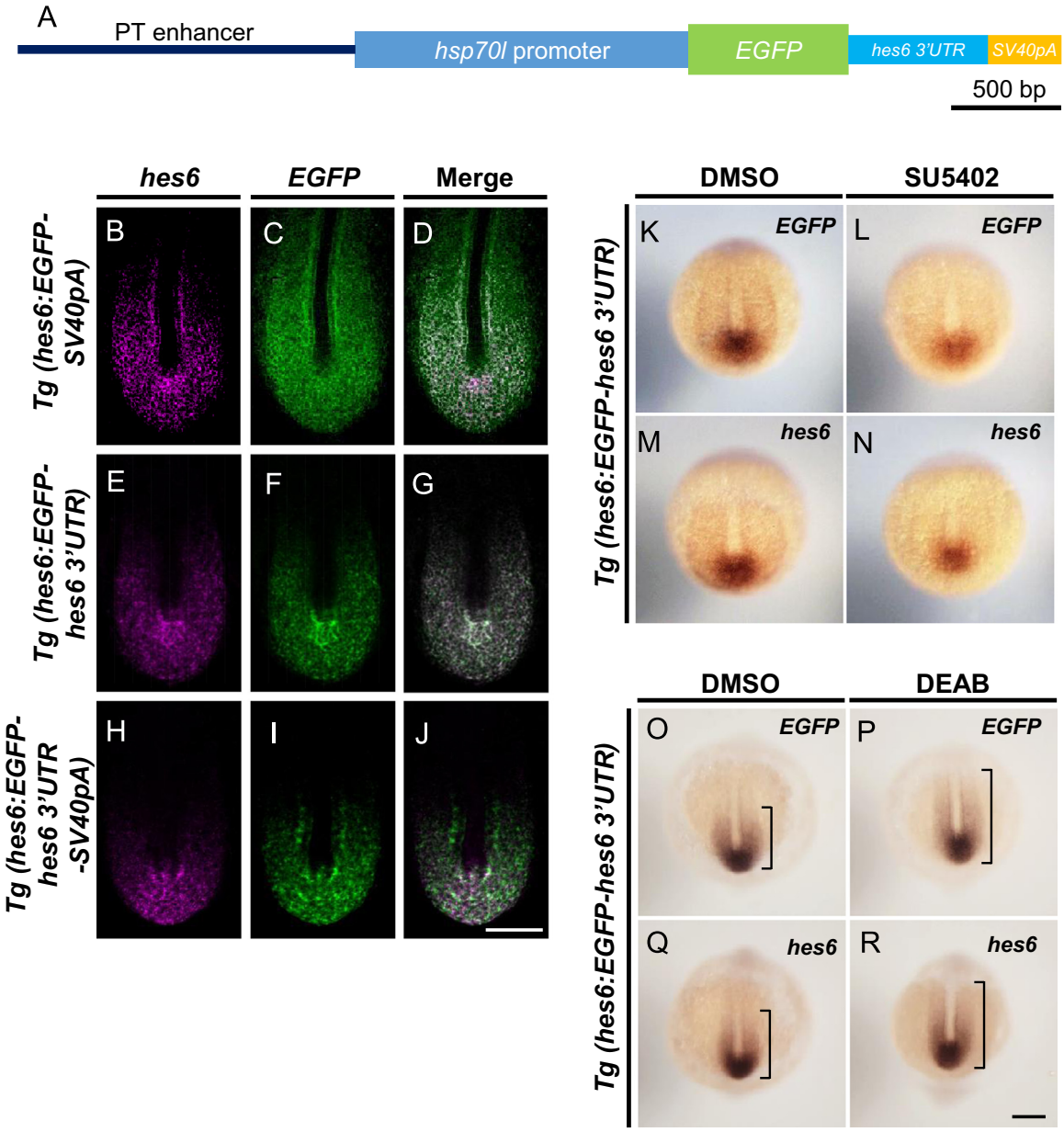


Fig. 7. PT enhancer with 3′UTR of *hes6* recapitulated a posterior-to-anterior gradient of *hes6* expression within the PSM. (A) *EGFP* reporter genes were used to generate the transgenic fish lines, *Tg(hes6:EGFP-hes6 3′UTR-SV40pA)*. To generate the reporter gene used in *Tg(hes6:EGFP-hes6 3′UTR-SV40pA)*, a DNA fragment corresponding to *hes6* 3′ UTR was inserted between *EGFP* and SV40pA in the reporter gene (shown in Fig. 2A). (B–J) Two-color fluorescent *in situ* hybridization. *Tg(hes6:EGFP-SV40pA)*, *Tg(hes6:EGFP-hes6 3′UTR)*, and *Tg(hes6:EGFP-hes6 3′UTR-SV40pA)* embryos were fixed at the 10–12-somite stage. Confocal images were taken of flat-mounted embryos after yolk removal. Endogenous expression of *hes6* is shown by magenta, and expression of *EGFP* mRNA is shown by green. Antisense probe for *hes6* is specific to the open reading frames of *hes6*. Scale bar in J, 50 μm. (K–R) Expression of *EGFP-hes6 3′UTR* mRNA was observed in transgenic embryos after treatment with SU5402 and DEAB; expression was similar to the endogenous expression of *hes6* mRNA. (K–N) Transient treatment of *Tg(hes6:EGFP-hes6 3′UTR)* embryos at the 1–2-somite stage with SU5402. *EGFP* expression was analyzed. (O–R) Treatment of *Tg(hes6:EGFP-hes6 3′UTR)* embryos from the sphere stage with DEAB. Embryos were fixed at the 10–12-somite stage and *EGFP* expression was examined. Dorsal views (K–R). Probes used for staining are shown at top-right of each panel. All images were taken at the same magnification. Scale bar in R is 200 μm.

(Diez del Corral et al., 2003). Although we cannot exclude the possibility that RA directly regulates the transcription of *hes6*, we presume that inhibition of RA synthesis results in the anterior expansion of the active domain of FGF signaling in the PSM, leading to the anterior expansion of *hes6* expression in the PSM.

Acknowledgments

We thank Drs Koichi Kawakami, Mami Matsuo-Takasaki, Makoto Kobayashi, and Hiroshi Sasaki for providing the plasmids. This work was supported partially by KAKENHI Grants-in-Aid for Scientific Research) to A.K. (22770208, 26430182) and K.Y. (23570247, 26440114) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.11.010.

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